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The Effect of Bangle (*Zingiber Cassumunar Roxb.*) Rhizome Chloroform Extract on Nitric Oxide and Reactive Oxygen Intermediate Secretions in Vitro

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Nurkhasanah
Faculty of Pharmacy
Ahmad Dahlan University
Yogyakarta, Indonesia
nurkhas@gmail.com

Nanik Sulistyani
Faculty of Pharmacy
Ahmad Dahlan University
Yogyakarta, Indonesia

Mifta Annisa Ghifarizi
Faculty of Pharmacy
Ahmad Dahlan University
Yogyakarta, Indonesia

Abstract—The immune system is needed by the body to maintain its integrity against the dangers caused by various substances in the environment. Immunomodulators are substances that can regulate the immune system and increase immunity. Bangle (*Zingiber cassumunar Roxb.*) Provides an immunomodulatory effect with an increase in phagocytic activity of macrophages and an increase in NO and ROI. This study aims to see whether there is an increase in NO and ROI secretion on macrophage of mice in vitro. Bangle rhizome was macerated using chloroform. Qualitative analysis by TLC method. The experimental study using macrophages isolated from male Balb mice divided into three concentrated variations 25, 50, 100 µg/ml. Quantitative analysis of NO secretion levels was done by Greiss reaction assay and analysis of ROI secretion levels by NBT reduction assay. The results showed that with the administration of bangle chloroform extract concentrations of 25, 50, 100 µg/ml had a significant effect ($p < 0.05$) on increasing NO secretion and ROI compared with the negative control group. In NO there was an increase in NO secretion levels with values in the control group, bangle chloroform extract groups concentrations of 25, 50, and 100 µg / ml respectively 11.8972µM, 17.7265µM, 19.4754µM and 22.3900µM. In ROI there was an increase in percentage ROI secretion with values in the control group, ERB groups concentrations of 25, 50, and 100 µg / ml respectively were 14.67%, 20.67%, 24%, and 27.3%. In conclusion, administration of bangle rhizome extract with concentrations 25, 50, 100 µg/ml has significant effect ($p < 0.05$) to increase the percentage of ROI secretion and NO secretion level of male Balb/C mice macrophages in vitro.

Keywords—bangle, immunomodulatory, NO secretion, ROI secretion

I. INTRODUCTION

The human body has a defense system to protect itself from attacks of foreign objects that can cause infection or tissue damage. The ability or system used by the body to maintain the integrity of the body as a protection against the dangers posed by the environment is called the immune system [7].

The immune system is needed by the body to maintain its integrity against the dangers caused by various substances in the environment [7]. Macrophages as effectors in the immune system, play a role in destroying germs or pathogens that will

damage the body, either through the mechanism of direct intracellular phagocytosis or indirect mechanisms by releasing Nitric oxide (NO), Reactive Oxygen Intermediate (ROI) and cytokines [24]. Increased immunity can be done by improving the function of the immune system using materials that stimulate the system called immunostimulators [7].

Immunomodulators are substances that can regulate the immune system, either in the form of restoring and repairing the immune system whose function is impaired or suppressing which functions are excessive [7]. One of the potential immunomodulatory medicinal plants is Bangle (*Zingiber cassumunar Roxb.*).

Bangle included in the Zingiberaceae group shows potential as an immunomodulator. Curcumin which is one of the compounds contained in the bangle rhizome is effective as an immunomodulatory agent. Curcumin provides immunomodulatory effects with an increase in phagocytic activity of macrophages [15]. Curcumin is able to increase activation of ROS (Reactive Oxygen Species) by activating signals involving Peroxisome Proliferator Activated Receptor Gamma (PPAR-γ) and Nrf-2 on macrophages and monocytes which causes an increase in CD36. Curcumin activates macrophages through the NF-κB pathway which releases various metabolites such as ROI and produces i-NOS which plays a role in NO synthesis. NO and ROI can improve the killing mechanism of macrophages [17].

According to Pramono (2013) curcumin dissolved in chloroform. Chloroform is a non-polar solvent so that substances dissolved in it are also non-polar substances according to the principle of like dissolve like. With the extraction using chloroform, it is expected that the active compounds contained in bangle and nonpolar in nature can be found perfectly.

II. MATERIAL AND METHOD

A. Research Design

This study was an experimental study with posttest control design by comparing four groups: control group, the chloroform extract group with concentrations of 25, 50, and 100µg/mL.

B. Plant Material

In this study, the sample used was rhizome bangle taken from the Bringharjo Market in Yogyakarta. Rhizome bangle is dried using an oven at a temperature of 50°C to obtain dry simplicia. The dried rhizomes are then blended. The 200 grams of rhizome bangle powder were macerated using chloroform until thick extracts were obtained.

C. TLC (Thin Layer Chromatography)

Test the presence of curcumin compounds using TLC with the stationary phase of silica gel GF254 and the mobile phase of chloroform: dichloromethane (3.25: 6.75) then seen on UV 366 nm and UV 254 nm.

D. Making Chloroform Extract Solution of Bangle

The 50 mg of chloroform extract of bangle in ependorf tubes. Then added dimethyl sulfoxide (DMSO) as much as 200 µL to dissolve the ethyl acetate fraction of bangle extract. The suspension of chloroform extract of bangle was made with concentrations of 25, 50, and 100 µg/mL.

E. Macrophage Isolation

In this study, using 2-3 months old male white mice Balb/C strain mice were obtained from Research Laboratory and Integrated Testing Gadjah Mada University Yogyakarta and has obtained ethical approval from the Ahmad Dahlan University Research Ethics Committee under the number 011804063. The mice were fasted for 10-12 hours, then narcosed with chloroform. After that, mice were sprayed with disinfectant solution and placed in the supine position. The abdominal skin was opened and cleaned the peritoneum sheath with 70% alcohol. The 10 ml of RPMI medium was injected into the peritoneum cavity and waited for 3 minutes. The peritoneal cavity fluid was aspirated with an injection syringe from a non-fat and distant part of the intestine. The aspirate was centrifuged at 1200 rpm, 4°C, for 10 min. The supernatant was removed, and the obtained macrophages resuspended by 1000 µl of complete medium. The number of cells was calculated by the hemocytometer with 10 µl of macrophages suspension [15].

F. Nitrite Oxide Test

The culture suspension macrophage of 122 µl was placed onto each well in 96 well micro plate and incubated for 24 hours in 5% CO₂ incubator at 37°C. After incubation for 24 hours, macrophage cells were treated by adding chloroform bangle extract solution with concentrations of 25 µg / mL, 50 µg / mL, and 100 µg / mL. Then re-incubated for 24 hours, then the 96-well microtiter plate containing the suspension from the cell culture was stored in the freezer. The culture suspension macrophage and the standard nitrite of 50 µl was placed onto each well in 96 well microplate. The sample was added by 50 µl greiss reagent, and let in room temperature for 15 min until the colour was changed. The absorbance of nitric oxide was observed on wavelength of 550 nm. Nitrite standard solution was used for calculating the concentration [16].

The standard nitrite with a concentration of 100 mM was made by dissolving 6.9 mg of sodium nitrite (Merck) in 10 mL aquadest. Stored at a temperature of 0-4°C, protected

from light, make a standard solution of various concentrations from the stock [16].

Greiss reagent was prepared by mixing Greiss A and Greiss B in the same amount. Greiss A was composed by of 0.1 g N-(1-naphthyl) ethylene diaminehydrochloride (NED) (Sigma N, 5889) in 100 ml of distilled water. Greiss B was prepared by mixing 1 g of sulfanilamide (Sigma N 5589) in 100 ml of 5% orthophosphoric acid. Greiss A and Greiss B must be stored in the dark place for protecting from direct light.

G. Reactive Oxygen Intermediate Test

For ROI secretion, the cells were grown with density of 5×10^5 cells/well into 6-well microplate. The cells was then incubated for 30 min in 5% CO₂ incubator with 37°C temperature, then 800 µl complete medium added into the susceptibility. Cell suspension for NO or ROI secretion test was incubated in 5% CO₂ incubator, 37°C for 24 hours. After incubation for 24 hours, macrophage cells were treated by adding chloroform bangle extract solution with concentrations of 25 µg / mL, 50 µg / mL, and 100 µg / mL, then incubated again for 24 hours in a 5% CO₂ incubator at 37°C. The supernatant is removed from the well, then added by 200 µL of NBT containing 125 ng/ ml of PMA in the middle of coverslip and incubated in 5% CO₂ incubator, 37°C for 60 min. The reagent was removed from the wells, and dried at room temperature. After cells were dry, cells were fixed with 1000 µl methanol for 30 seconds. After drying, cells were added by 200 µl neutral red spultion 2% for 15 minutes and dried at room temperature. After drying, cellswere rinsed with distilled water. The percentage of macrophage cells showing NBT reduction was calculated from about 100 cells examined with a 400 times magnification of light microscope.

H. Data Analysis

1) Nitrit Oxide

NO secretion was tested by the Griess reaction assay which produced a pink color which was then measured by ELISA reader. Secretion of NO by macrophages was calculated by comparing with the standard curve NO (standard nitrite solution)[16].

2) Reactive Oxygen Intermediate Test

The percentage of macrophage cells showing NBT reduction was calculated from about 100 cells examined with a 400 times magnification of light microscope.

3) Statistics Test

Normality test was carried out using this test Saphiro-Wilk, with a data amount of less than 50. Continued variance homogeneity test using the Levene test. If the normal distribution test and the variance homogeneity test are homogeneous and normally distributed, the test is continued with parametric one-way variance analysis, ANOVA and LSD test.

III. RESULTS AND DISCUSSION

A. Qualitative Analysis of Chloroform Extract oc Bangle

The extraction method used in this study is maceration. Maseration is the process of extracting simplicia using a

solvent with stirring at room temperature. This study only conducted qualitative tests on curcumin compounds contained in bangle rhizomes. Rhizome bangle is a Zingiberaceae plant group that has many chemical compounds. This is evidenced from the results of research that states that bangle rhizome contains saponins, flavonoids, essential oils, tannins, and glycosides. Not only that, the bangle rhizome is also known to contain bioactive curcumin which can be used as an immune enhancing agent [17]. For this reason, it is important to carry out the TLC test as proof that the bangle rhizome contains the active compound which has the ability to increase endurance. The results of TLC observed under UV 254 and 366 nm rays can be seen in figure 1.

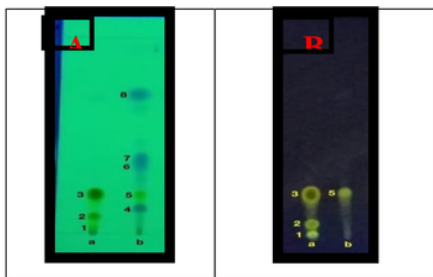


Fig. 1. Thin layer chromatogram profile with the mobile phase of chloroform: dichloromethane in UV 254 nm (A) dan 365 nm (B): a. curcumin standard, b. chloroform extract of bangle.

The results of the chromatogram extract of chloroform rhizome bangle under UV 254 nm light produced 5 spots. According to research from Ravi et al. (2011) showed that there were 5 spots produced by elution of bangle rhizome extract with the same mobile phase composition. From the results of the standard research curcumin has yellow spots under 254 nm UV light with its Rf value is 0.23 and for the chloroform extract sample the bangle rhizome one of them has yellow spots on UV 254 nm and Rf values that are the same as the standard curcumin (0.23). This shows that the presence of curcumin in the chloroform extract is rhizome bangle because the Rf value of the extract is the same as the standard Rf value. The results of the calculation of the chloroform bangle extract Rf and the curcumin standard can be seen in Table I.

TABLE I. Rf RESULTS OF CHLOROFORM EXTRACT OF BANGLE AND CURCUMIN STANDARD

Spot	Ethyl Acetate Fraction		Rf	Curcumin Standard		Rf
	UV 254	UV 366		UV 254	UV 366	
1	Blue-Purple		0,15	Yellow-Brown	Yellow	0,23
2	Yellow-Brown	yellow	0,23	Yellow-Brown	yellow	0,1
3	Blue-Purple		0,34	Yellow-Brown	Yellow	0,23
4	Blue-Purple		0,38			
5	Blue-Purple		0,75			

B. Result of Nitric Oxide test

The NO macrophage secretion test is one of the testing of immune activity. Beginning with phagocytosis of

macrophages which is the first stage in the response to the attack of microorganisms and activates an increase in the innate immune response. During the process of phagocytosis, activated macrophages produce reactive oxygen species (ROS) such as NO and H₂O₂ [1].

The ability of peritoneal macrophages to secrete Oxyda Nitrite (NO) was measured by ELISA Reader. Previously the samples were reacted first with the Griess reagent (diazotization reaction). This is one of the simple NO detection methods with the principle of colorimetry. In this method the nitrite is reacted with sulfanilamide in an acidic state to form a diazonium salt, which in turn reacts with coupling reagents (N-naphthyl-ethylendiamine (NED)) into a stable azo form and produces an intense pink color. Then read by ELISA reader at wavelengths with a range of 550 nm (Nurkhasanah et al., 2017). The working principle of ELISA reader is spectrophotometry that can read absorbance with many samples and at least 100 µL the volume of solution, the purpose of reading with ELISA reader so that standard samples and nitrites in the microtiter plate 96 well can be read all at once.

Before calculating NO secretion a standard curve of standard nitrite solution with various concentrations is made. Linear regression obtained between the concentration versus absorbance of standard nitrite is used to calculate the secretion levels of NO [17] ples. The graph of the raw curve can be seen in Figure 2 with a linear regression equation $y = 0.0063x + 0.0435$ so that the average level of NO obtained as presented in Table II.

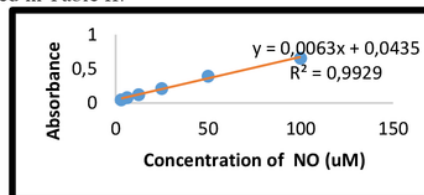


Fig. 2. The standard nitrite curve.

TABLE II. THE NITRITE OXIDE SECRETION LEVEL OF MACROPHAGE WHICH TREATED BY CHLOROFORM EXTRACT OF BANGLE RHIZOME IN VITRO

Treatment group	NO secretion level \pm SD (μ M)
Negative control	11,8972 \pm 0,40
Extract 25 μ g/ml	17,7265 \pm 1,24*
Extract 50 μ g/ml	19,4754 \pm 0,69*
Extract 100 μ g/ml	22,3900 \pm 1,69*

(*) = significantly different from the control group

The results of the study showed that the mean NO secretions of all groups. The lowest NO secretion level was indicated by the control group (11.8972) while the highest NO secretion level was shown in the group of extracts of rhizome bangle concentration of 100 µg / ml (22.3900). The mean levels of NO secretion of mice macrophages given rhizome bangle extract can be seen in Table II. The concentration of group secretion by giving variation of bangle root extract concentration 25, 50, 100 µg / ml was higher than the secretion level of the control group. Increasing NO levels by administering rhizome bangle extract proves that rhizome bangle extract has the potential as an immunostimulant agent.

The results of the statistical analysis showed that in the bangle rhizome extract group concentrations of 25, 50, 100 $\mu\text{g/ml}$ had significantly different NO levels compared to the control group. Namely the group that was given only cells and medium but was not given an extract of rhizome bangle. This shows the administration of bangle rhizome extract with concentrations of 25, 50, 100 $\mu\text{g/ml}$ in mice macrophages can provide potential immunomodulatory effects on mice by increasing NO secretion in peritoneal macrophage cells. The comparison between groups of bangle rhizome extract concentrations of 100 $\mu\text{g/ml}$ with 25 bangle rhizome extract groups and 50 $\mu\text{g/ml}$ extracts were significantly different [23]. However, in the group of extracts of rhizome bangle the concentration of 25 $\mu\text{g/ml}$ compared to the group of extracts of rhizome bangle concentration of 50 $\mu\text{g/ml}$, the results were not significantly different. It can be concluded that in the bangle rhizome extract group a concentration of 25 $\mu\text{g/ml}$ with a group of bangle rhizome extract concentrations of 50 $\mu\text{g/ml}$ with an increase in the given dose did not cause the expected increase in effect. Previous results of Nurmasari (2013) giving stimulation of bangle ethanol extract for 14 days and therapy for 4 days dose 18.08 mg / 20gBB can increase NO expenditure compared to groups without therapy. Curcumin is able to increase ROS by activating signals involving Peroxisome Proliferator Activated Receptor Gamma (PPAR- γ) and Nrf-2 on macrophages and monocytes that cause an increase in CD36 so that it can increase phagocytosis of macrophages. Phagocytosis of macrophages is the first line of defense against various types of infection. Activated macrophages receive signals from IFN- γ to produce inducible Nitric Oxide Synthase (iNOS). The enzyme catalyzes the conversion of L-arginine to L-citrulline which produces Nitric Oxide gas. Increased production of Nitric Oxide is associated with increased macrophage activity as phagocytic cells. Curcumin activates macrophages through the NF- κ B pathway which releases various metabolites such as ROI and produces i-NOS which plays a role in the synthesis of Nitrite Oxide (NO). NO and ROI can improve the killing mechanism of macrophages. In Chairul's (2009) study 3 components of phenylbutanoid isolation from methanol extract of rhizome bangle showed immunostimulatory activity with increased phagocytic activity of macrophages in vitro. Phenylbutanoid shows antioxidant activity by counteracting free radicals and can increase phagocytic activity (Nurkhasanah et al., 2017).

C. Result of Reactive Oxygen Intermediate Test

ROI is an oxidative product of macrophages that plays an important role in the mechanism of destruction / killing of bacteria whose activities are induced by IFN- γ and TNF- α cytokines [23]. Peritoneal macrophage activity in secreting ROI was observed using the NBT Nitroblue Tetrazolium (NBT) Reduction assay reduction test containing Phorbol 12-Myristate 13-Acetate (PMA). PMA will stimulate macrophages to secrete ROI. The presence of ROI (superoxide anion, O $_2^-$) shows an increase in respiration and causes NBT to be reduced so as to form a formazan precipitate that is not soluble in blackish blue [15]. The microscopic results of peritoneal macrophages that secrete ROI and those that do not secrete can be seen in Figure 3.



Fig. 3. Microscopic results of macrophages after administration of chloroform extract rhizome bangle for 24 hours in vitro at 400x magnification which secretes ROI (blue arrow) and which does not secrete ROI (green arrow).

Formazan deposits in macrophages show macrophages secrete ROI, meaning that the more macrophages that form formazan deposits, the higher the ROI secretion activity by macrophages. Formazan which is not dissolved can be observed with a microscope 400x magnification. Calculations were performed on 100 macrophages seen under a light microscope.

TABLE III. THE MEAN LEVELS OF ROI SECRETION OF MACROPHAGE WHICH TREATED BY CHLOROFORM EXTRACT OF BBANGLE RHIZOME IN VITRO

Group	Average Secretion Level ROI \pm SD
Negative control	14.67% \pm 0.57
Extract 25 $\mu\text{g/ml}$	20.67% \pm 2.31*
Extract 50 $\mu\text{g/ml}$	24.00% \pm 1.00*
Extract 100 $\mu\text{g/ml}$	27.30% \pm 2.52*

(*) = significantly different from the control group

Table III shows the lowest average percentage of ROI secretion shown by the control group (14.67%), while the highest percentage of ROI secretion was shown by the group of rhizome extract bangle concentration of 100 $\mu\text{g/ml}$ (27.30%). Percentage of secretion group by giving variation of bangle rhizome extract concentration 25, 50, 100 $\mu\text{g/ml}$ was higher than percent of control group secretion. Increasing the percentage of ROI secretion proves that rhizome bangle extract has the potential as an immunomodulatory agent.

The value of NBT levels is proportional to ROI secretion. The higher the value of ROI secretion shows the greater free radical product used to fight pathogens. Giving rhizome bangle extract as an immunomodulator can stimulate the body's immune system, marked by an increase in ROI secretion. The Mann-Whitney test results showed the group of bangle rhizome extract concentrations of 25, 50, 100 $\mu\text{g/ml}$ had a significantly different percentage of ROI compared to the control group. That is a group that is only given a complete cell and medium but is not given a rhizome extract of bangle. This shows the administration of bangle rhizome extract with a concentration of 25, 50, 100 $\mu\text{g/ml}$ in mice macrophages influences the increase in ROI secretion, rhizome bangle extract can have an effect as an immunostimulant.

Comparison between groups of bangle rhizome extract concentrations of 25 $\mu\text{g/ml}$ with 50 bangle rhizome extract group and 100 $\mu\text{g/ml}$ results were significantly different. However, in the bangle rhizome extract group the concentration of 50 $\mu\text{g/ml}$ had a percentage of ROI that was not significantly different compared to the group of bangle rhizome extract concentrations of 100 $\mu\text{g/ml}$. It can be concluded that there is a significant effect between the group of bangle rhizome extract concentration of 25 $\mu\text{g/ml}$ and rhizome extract of bangle concentration of 50 $\mu\text{g/ml}$ and extract of rhizome bangle concentration of 100 $\mu\text{g/ml}$. However, in the group of bangle rhizome extract

concentrations of 50 µg / ml with a group of bangle rhizome extract concentrations of 100 µg / ml it was concluded that there was no significant effect. The average difference in the percentage of ROI secretion in the ERB treatment groups 5, 10 and 20 mg / 20gBB is called an independent dose, where the increase in the dose given does not cause the expected increase in effect. Possibly caused by macrophages that have microbicidal molecules, namely NO and ROI produced by lysosomes in severe inflammatory reactions that are very active in killing microbes [15]. Previous results showed that administration of bangle rhizome extract dose 5 and 20 mg / 20gBB per day for 7 days increased the percentage of ROI secretion compared to groups not given rhizome bangle extract on peritoneal macrophages of male Swiss mice induced by LPS 14 µg / 20gBB [15]. This is due to the content of the rhizome of bangle curcumin which has the potential as an immunostimulant agent. Curcumin is able to increase ROS by activating signals involving Peroxisome Proliferator Activated Receptor Gamma (PPAR-γ) and Nrf-2 on macrophages and monocytes which causes an increase in CD36. Curcumin activates macrophages through the NF-κB pathway which releases various metabolites such as ROI and produces i-NOS which plays a role in the synthesis of Nitrite Oxide (NO) [17]. NO and ROI can improve the killing mechanism of macrophages.

IV. CONCLUSION

The administration of bangle rhizome extract concentrations of 25, 50, 100 µg / ml can increase NO secretion and ROI of peritoneal macrophages in male Balb / C mice in vitro. NO secretion and ROI of macrophages with the administration of chloroform extracts of bangle roots concentrations of 25, 50, and 100 µg / ml increased with increasing concentration, in this study NO secretion and ROI were highest at concentrations of 100 µg / ml.

ACKNOWLEDGMENT

Thanks to Technology Research and Higher Education for funding through the 2018 Postgraduate Team Research Scheme.

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